

# Dissecting Location-Specific Signaling Pathway

Activity in the Neurogenic Niche

By

Justin Evans

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Approved:

Rebecca Ihrie, Ph.D.

Chin Chiang, M.D., Ph.D.

Jin Chen, Ph.D.

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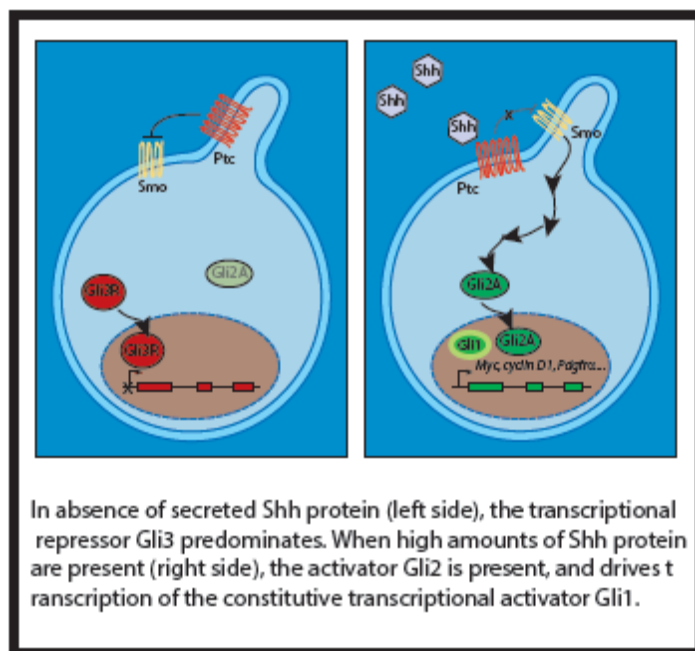
## CHAPTER 1

# DISSECTING LOCATION-SPECIFIC SIGNALING PATHWAY ACTIVITY IN THE NEUROGENIC NICHE

### Introduction

The adult mammalian brain hosts two regions of slow-dividing neural stem cells that generate new neurons throughout life<sup>7, 8, 10, 11</sup>. One of these regions, the subventricular zone (SVZ), is composed of three populations of cells: (1) neural stem cells, which give rise to rapidly dividing (2) transit amplifying cells, which in turn generate (3) immature neuroblasts that are destined for the olfactory bulb<sup>8, 11, 15</sup>. Within the olfactory bulb, these neuroblasts supplement the interneuron granular layer and periglomerular cell populations<sup>8, 10</sup>. Recently, it has been revealed that significant heterogeneity exists in the stem cell population of the SVZ<sup>1, 8-10, 17, 24</sup>. Rather than generating all possible neuronal types, as was previously supposed, it is now clear that a stem cell in a specific region generates a specific subset of interneurons in the olfactory bulb<sup>8, 10</sup>. This tight regulation of progeny fate is encoded at least in part through cell-intrinsic mechanisms.

Surprisingly, the first signaling pathway that has been found to regulate specific progeny fates in the SVZ is driven by a secreted ligand<sup>8</sup>. Within the SVZ, ventral neural stem cells have high Sonic hedgehog (Shh) pathway activation, while dorsal neural stem cells lack pathway activation but express many of the upstream pathway components<sup>8, 17</sup>. Surprisingly, the removal of the Shh receptor complex Smo, which is required for signaling, in SVZ neural stem cells at postnatal day 60 compromises proliferation and neuron production in both the dorsal and ventral regions<sup>2, 17</sup>. This suggests that the Shh pathway is required for neurogenesis, but only low levels of pathway activation maybe required for neuron production in the dorsal region.



Gli3 is a DNA-binding transcription factor that acts as a negative mediator of Shh signaling. In vertebrates, the zinc-finger transcription factor Gli3 undergoes proteolysis to its N-terminal repressor form. Expression of the Gli3 N-terminal repressor acts primarily as a negative mediator of

Shh signaling.<sup>4, 12-14, 23</sup> The expression of the Gli3 N-terminal repressor has been shown to be critical for Dorsal/Ventral patterning of the developing neural tube and for progenitor proliferation and cell fate in the cortex<sup>12, 22</sup>. Gli3 expression studies in the postnatal forebrain have demonstrated that Gli3 expression levels are elevated in the

dorsal region of the SVZ<sup>17</sup>. In this study, we focus on the role of Gli3 in proliferation and fate specification of dorsal SVZ-derived neurons using conditional ablation to remove Gli3 in a forebrain stem cell niche after Dorsal/Ventral patterning is complete.

## Experimental Procedures

### Animals

The following mouse lines were used and genotyped as previously described: Gli3<sup>lox</sup> (Huang et al Dev Dyn, 2008.) and Ai14<sup>lox</sup> (Jackson Labs). Mice of either sex were used for the analysis. To label proliferating cells, BrdU (10 mg ml<sup>-1</sup> solution, 50 mg kg<sup>-1</sup> body weight, Sigma) was injected intraperitoneally. Experiments were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

### Injections and Adenovirus Preparation

Ad:GFAPpCre into was injected into dorsal and ventral adult SVZ as described (Merkle et al., 2007) using 50 nl of virus. Injections used the following coordinates: dorsal SVZ —0.5 anterior, 3.2 lateral, 1.8 deep, needle at 45 angle; ventral SVZ—0.5 anterior, 4.6 lateral, 3.3 deep, needle at 45 angle, the x and y coordinates were zeroed at bregma, and the z coordinate was zeroed at the pial surface.

## Immunostaining

Immunostainings were carried out on paraformaldehyde-fixed 50 micron free floating Vibratome sections according to standard procedures. Sections were blocked with 1% goat serum plus 0.10% Triton X-100(Sigma) in PBS for 30 min at 25 C, before primary antibody incubation at 4 C overnight. Primary antibodies used were mouse anti-GFAP (1:1000, Chemicon), chicken anti-GFAP (1:1000, Abcam), rabbit anti-doublecortin (1:1000, Cell Signaling), Guinea pig anti-doublecortin (1:1000, Cell Signaling), chicken anti-GFP (1:1000, Aves Labs), rabbit anti-RFP (MBL), rabbit anti-tyrosine hydroxylase (1:1000, Pel-Freez Biologicals), monoclonal anti-calbindin D28k(1:1000, Sigma), rabbit anti-calretinin (1:1000, Swant), rat anti-BrdU(1:100, Abcam), and rabbit anti-calbindin D28k (1:1000, Swant). The secondary antibodies used were conjugated to AlexaFluor dyes (Invitrogen/Molecular Probes) and nuclei were counterstained with DAPI (Sigma).

## Microscopic Analysis and Quantification

Fluorescent staining was visualized using a LSM 710 Meta Inverted confocal microscope or a Nikon AZ 100 M widefield epifluorescent scope. Measurements of olfactory interneuron localization were carried out using a custom-designed script for Olfactory bulb scoring plugin in Metamorph, with normalization to granular layer width carried out as described (Merkle et al., 2007). Data were quantified and analyzed using GraphPad Prism 5.

## Western Blot Analysis

Tissue was micro-dissected from the dorsal wedge area, ventral SVZ, and corpus callosum of wildtype mice. Tissue extracts were prepared by lysing the tissue with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing freshly added phosphatase inhibitor cocktails I and II (P-2850 and P-5726; Sigma), Complete protease inhibitor cocktail (Roche), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) on ice for 30 min. Insoluble debris was removed by centrifugation at 16,000 g for 20 min at 4°C.

Lysate concentrations were determined with the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL) and compared to BSA standards in RIPA buffer, and the samples were equalized with RIPA buffer. 30µg aliquots of the lysates were separated on a 4 to 10% Tris-glycine SDS-PAGE with Precision Plus protein standards (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (all reagents from Invitrogen, Carlsbad, CA). Membranes were incubated with 2 to 5µg/ml anti-Gli antibodies in 5% (wt/vol) milk in Tris-buffered saline–0.05% Tween 20 followed by horseradish peroxidase(HRP)-conjugated secondary antibodies. Endogenous Gli bands were visualized with a rabbit monoclonal antibody against Gli3, gli1 antibody (1:500, Cell Signaling), and tubulin loading controls (1:10,000 dilution of 1A2 MAb; Sigma T9028) were visualized with ECL reagent(GE HealthCare).



Figure 1: Gli1 is Higher in Ventral Progenitors, and Gli3 Repressor is Increased in Dorsal Progenitors

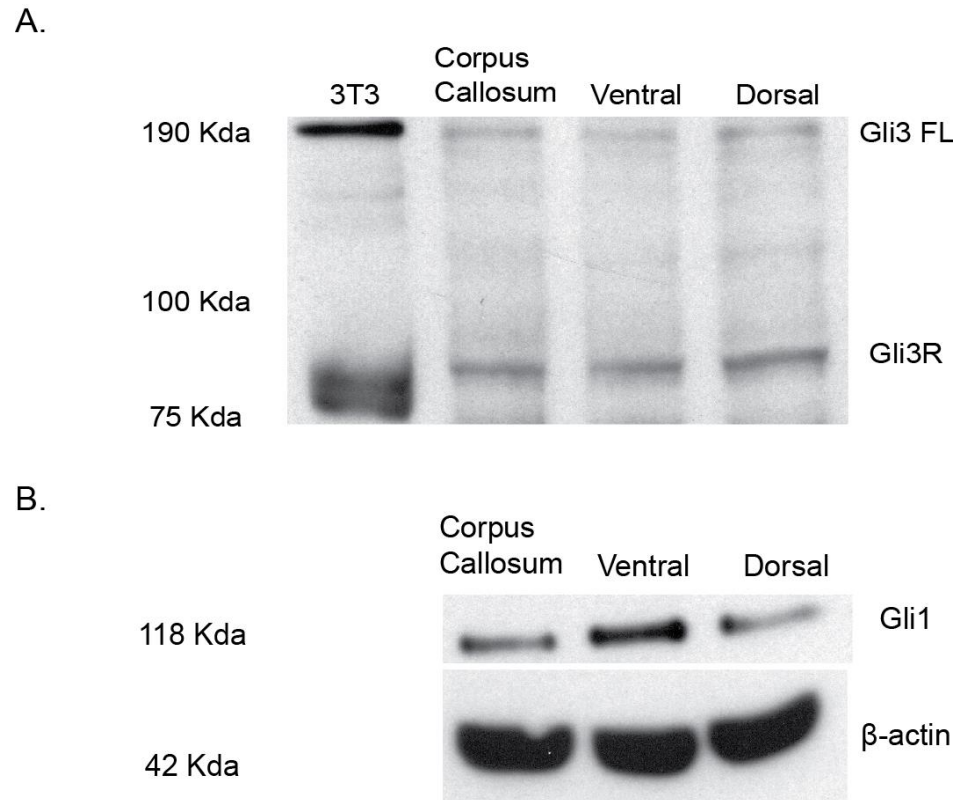


Figure 1: Gli1 is Higher in Ventral Progenitors, and Gli3 Repressor is Increased in Dorsal Progenitors. Lysates from 3 adult WT mice were micro-dissected and collected from the Dorsal and Ventral SVZ. These samples were then blotted for Gli1 and Gli3. A. Western blotting shows an increase of Gli3 Repressor protein in the Dorsal SVZ compared with the Ventral SVZ. B. Western blotting shows a marked increase of Gli1 protein in the Ventral SVZ compared with the Dorsal SVZ.

Figure 2: Loss of Gli3 Results in Aberrant Positioning of Dorsal-Derived Progeny in the OB

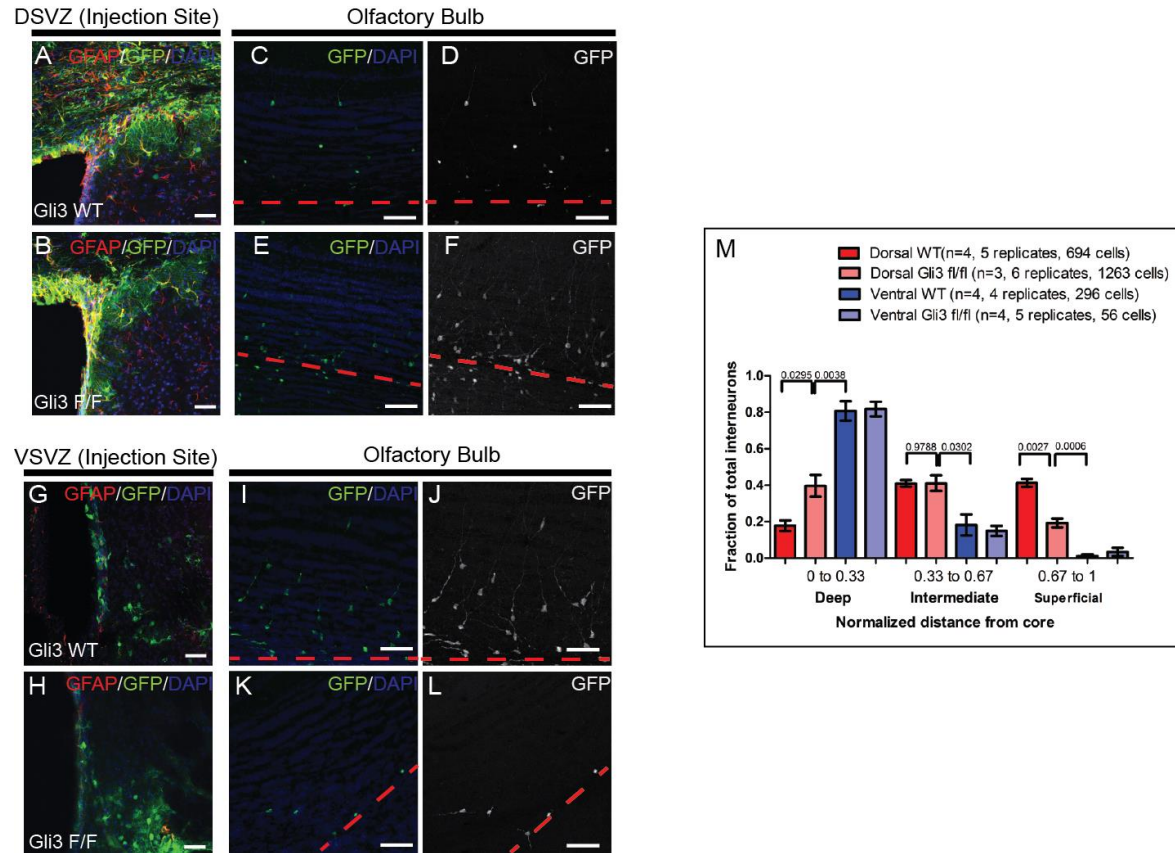


Figure 2. Loss of Gli3 Results in Aberrant Positioning of Dorsal-Derived Progeny in the OB. (A-L) Ad:GFAPp-Cre was injected into dorsal and ventral SVZ of Gli3  $+/+$  and Gli3  $fl/fl$  conditional animals also carrying a conditional GFP reporter and brains were collected one month later to analyze labeled progeny. The GFP reporter labels cells that have undergone recombination. GFP staining was combined with staining of GFAP within the SVZ (A, B, G and H) to confirm the site of virus injection. Dorsal injections in control animals primarily generated superficial labeled cells (C-D) that are distant from the core of the OB (indicated by the dashed line). Injections in the dorsal SVZ of Gli3 conditional animals gave rise to a greater number of deep cells located close to the core of the OB (E-F). However, the distribution of cells resulting from dorsal Gli3 loss did not fully recapitulate the distribution seen from ventral injections in either genotype (compare light red and blue bars in M). M. Quantification of the distribution of labeled Gli3 wildtype and Gli3 floxed animals after ad:GFAPp-Cre viral injection. Dorsal injection into wildtype (bright red bar) results in cells that are mostly located distant from the core of the OB. However, upon Gli3 ablation (pale blue bar) increased numbers of cells are seen in the deep region (pale red bar). Ventral injection into Gli3 floxed animals shows that the ablation of Gli3 has no effect on cell distribution (comparing bright and pale blue). Error bars, mean  $\pm$  SEM from the indicated number of mice. Scale bars, 50  $\mu$ m.

Figure 3: Loss of Gli3 Results in a Loss of TH expression in Dorsal-Derived OB Neurons

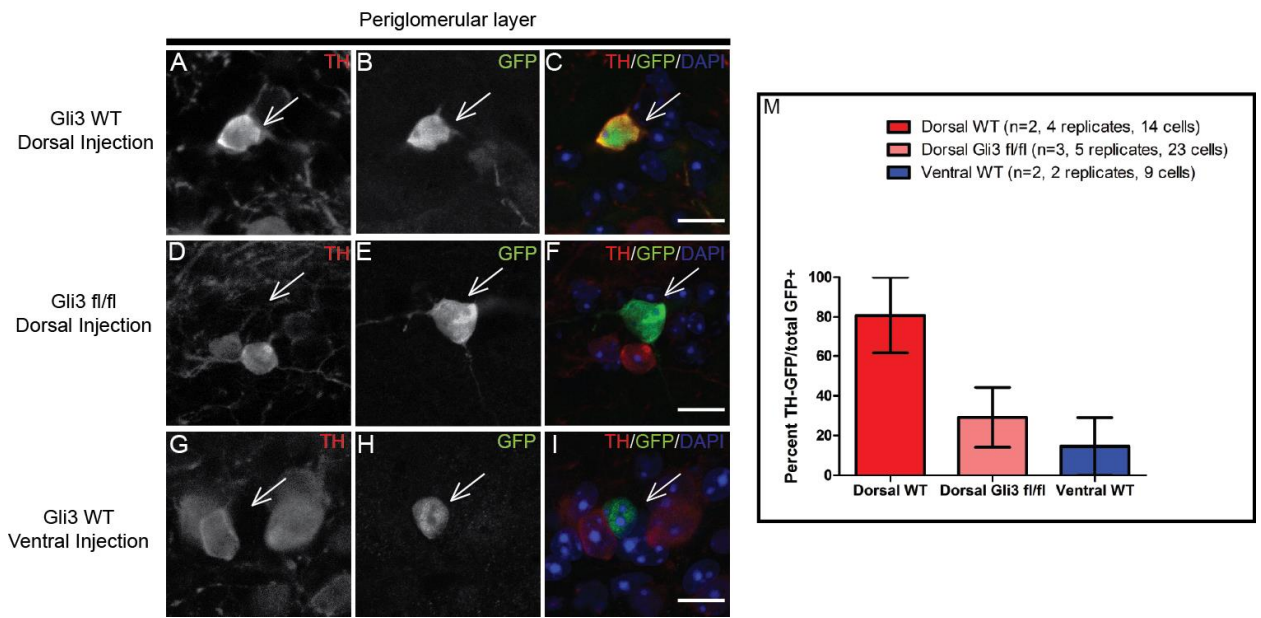


Figure 3. Loss of Gli3 Results in a Loss of TH expression in Dorsal-Derived OB Neurons (A-I) Ad:GFAPP-Cre was injected into dorsal and ventral SVZ of Gli3  $+/+$  and Gli3  $fl/fl$  conditional animals and brains were collected one month later to analyze labeled progeny. GFP staining was combined with staining of TH and Calbindin, which are markers of dorsal-derived and ventral-derived cells respectively. Dorsal injections into control animals primarily generate TH-positive cells in the periglomerular layer (A-C). The production of TH-positive cells in the periglomerular layer is decreased in the absence of Gli3, similar to rates as those seen in ventral-derived periglomerular layer cells (G-I). M. Quantification of the GFP/TH co-expressing cells in Gli3 wildtype and Gli3 floxed animals after Ad:GFAPP-Cre viral injection. Injections into the dorsal region of Gli3 control animals primarily generate TH-positive cells. However, injections into the dorsal region of Gli3 floxed animals result in periglomerular cells that largely do not express TH, similar to injections in the ventral region of wildtype animals (comparing pale blue bar and pale orange bar). Error bars, mean  $\pm$  SEM from the indicated number of mice. Scale bars, 10  $\mu$ m.

Figure 4. Loss of Gli2-Gli3 Rescues Granule Cell Body Localization

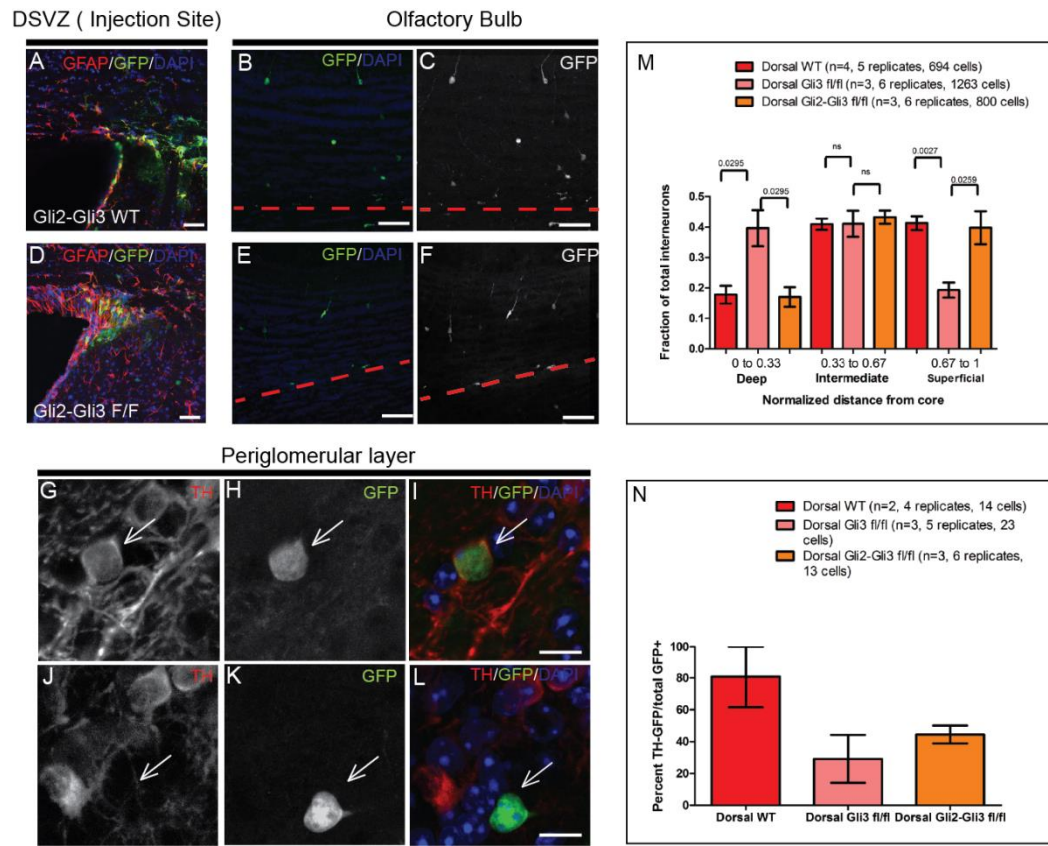


Figure 4. Loss of Gli2-Gli3 Rescues Granule Cell Body Localization (A-L) Ad:GFAPP-Cre was injected into dorsal and ventral SVZ of Gli2-Gli3 fl/fl double-conditional animals. Brains were collected one month later to analyze labeled progeny. GFP staining was combined with staining of GFAP within the SVZ (A, B, G and H) to confirm the site of virus injection. Dorsal injections into control animals primarily generate superficial labeled cells (C-D) that are distant from the core of the OB (indicated by the dashed line). Gli2-Gli3 ablation in the dorsal SVZ (E-F) results in labeled superficial cells in a similar distribution to those seen in littermate controls after dorsal injections. (C-D). GFP staining was combined with staining of TH and Calbindin, which are markers of dorsal-derived and ventral-derived cells respectively. Dorsal injections into control wildtype animals primarily generate TH-positive cells in the periglomerular layer (G-I). The production of TH-positive cells in the periglomerular layer is remains diminished in the absence of Gli2 and Gli3(J-L), when compared to dorsal-derived periglomerular layer cells from control animals (G-I). M. Quantification of the distribution of labeled cells in Gli2-Gli3 wildtype and Gli2-Gli3 floxed animals after Ad:GFAPP-Cre viral injection. Dorsal injection into Gli2-Gli3 wildtype (pale red bar) results in cells that are mostly located distant from the core of the OB. However, upon Gli3 ablation (pale blue bar) there is a shift in cells to the deep region, which is not observed when both Gli2 and Gli3 are ablated (pale orange bar). N. Quantification of the GFP/TH co-expressing cells in Gli2-Gli3 wildtype and Gli2-Gli3 floxed animals after ad:GFAPP-Cre viral injection. Injections into the dorsal region of Gli2-Gli3 control animals (pale red) primarily generate TH-positive cells. However, injections into the dorsal region of Gli2-Gli3 floxed animals and Gli3 floxed animals have a decreased population of TH positive cells, when compared to wildtype animals (pale red, blue and orange). Scale bars, 50  $\mu$ m in figures A-F and 10  $\mu$ m in figures G-L.

## Results

### Gli3 Repressor is increased in DSVZ

Mapping of the beta galactosidase activity in *Gli3-nlacZ* mice has shown that high levels of Gli3 expression are restricted to the dorsal region of the SVZ<sup>17</sup>. However, it is known that the Gli3 protein must undergo proteolysis to its repressor form to function as a negative regulator of the Shh pathway<sup>14, 23</sup>. The expression of Gli3R protein in the neural stem cell (NSC) and progenitor population of the SVZ has not been characterized. We first examined whether the Gli3 repressor was enriched in the dorsal region of the SVZ compared to the ventral region, which is known to have high pathway activation. Using lysates generated from micro-dissections of dorsal SVZ, ventral SVZ, and corpus callosum, we conducted Western blot analysis. We were able to determine that there were higher levels of the Gli3 repressor in the dorsal SVZ when compared to the ventral SVZ (Figures 1A). To determine whether this pattern correlates to levels of Shh activity, we also measured Gli1, which is a readout for high pathway activation. Gli1 levels were lower in the dorsal SVZ and higher in the ventral SVZ (Figure 1B). These results indicate that *Gli3* is higher in the dorsal NSC in the SVZ.

### Loss of Gli3 Results in Aberrant Positioning of Dorsal-Derived Progeny in the OB

Next, we investigated whether the Gli3 repressor was required for fate specification of dorsal-derived progeny. Dorsal progeny migrate anteriorly through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they integrate into two regions: 1) the superficial region of the granular layer; or 2) the periglomerular layer,

where they expression the tyrosine hydroxylase protein <sup>8, 10, 17</sup>. To determine whether Gli3 was required for the fate specification of dorsal-derived progeny, we injected conditional *Gli3<sup>fl/fl</sup>*; CAG mice and littermate controls at P60 with Ad:GFAPp-Cre in the dorsal or ventral region to ablate Gli3 in the GFAP expressing NSC. Mice were collected a month later and targeting was verified (Figure 2A,B,G, and H). Next, the OB was analyzed for the effects of Gli3 loss. The loss of Gli3 resulted in an aberrant shift of dorsal-derived progeny from the superficial region to a region closer to the core of the OB, where ventral-derived progeny reside (Figure 2C-J). Interestingly, the loss of Gli3 in the ventral region seemed to have no effect on the positioning of progeny originating from this region (Figure 2K-L). Taken together, our results suggest that the Gli3 mutants have a bias to producing deep layer interneurons at the expense of superficial interneurons.

#### Loss of Gli3 Results in a Loss of Dorsal-Derived TH-Expressing OB Neurons

Since the loss of Gli3 in dorsal SVZ resulted in the generation of progeny that integrated into the deep granular layer we next investigated whether the loss of Gli3 had an effect on production of dorsal-derived progeny in the periglomerular layer. Dorsal-derived progeny can be identified in the periglomerular layer by the expression of tyrosine hydroxylase (TH)<sup>10</sup>. To determine whether the loss of Gli3 resulted in changes of fate specification, we analyzed the expression of TH in Gli3 mutants and littermate controls. We found that in the littermate controls dorsal-derived periglomerular cells express TH, but this expression was lost when Gli3 was ablated (Figure A-F).

Next, when Gli3 is ablated dorsal-derived progeny in the granular layer shift to a

more ventral-like phenotype, residing in the deep region, so we decided to investigate whether dorsal-derived PGCs respecify to ventral-like PGCs. It has been determined that ventral-derived PGCs express the calcium binding protein calbindin. Interestingly, we were not able to detect increased frequency of calbindin expression in the Gli3 mutants (data not shown).

### Loss of Gli2-Gli3 Partially Rescues Granule Cell Body Localization

Previous studies in the neural tube have shown that in the absence of Shh signaling, Gli2 is maintained at a low level and Gli3 is continually cleaved into its repressive form<sup>5, 6, 12, 20, 21</sup>. Our previous results suggest that the Gli3 repressor is critical for the fate specification of dorsal-derived progeny. Since it is known that in the absence of Gli3, Gli2 may activate the Shh pathway, we wanted to determine whether Gli2 was required for the phenotype observed in Gli3 mutants. To determine whether Gli2 drives fate specification of dorsal-derived progeny in the absence of Gli3 we injected conditional Gli2-Gli3; CAG mice and littermate controls at P60 with Ad:GFAPp-Cre in the dorsal region to ablate Gli2-Gli3 in the GFAP expressing NSC. Analysis of the double mutant (Gli2-Gli3) progeny within the granular layer revealed that in the absence of both Gli2 and Gli3 granule interneuron cell bodies predominantly localize to the superficial region (Figure 4E-F), similar to that of the littermate controls (Figure 4B-C).

To further investigate the contributions of Gli2 in the absence of Gli3, we analyzed the dorsal-derived PGCs. Surprisingly, the TH expression in the double mutant PGC population was not restored (Figure G-L), when compared to the littermate control animals. These results suggest that Gli2 drives the fate of dorsal-derived progeny in the



absence of Gli3, and that removing both transcription factors partially , but not completely, restores their identity.

## Discussion

We demonstrate that the hedgehog pathway has a critical role in fate specification in both the dorsal and ventral region of the SVZ. It was shown previously that the ventral region of the SVZ has high pathway activation and that this activation is required for the production of ventral-derived progeny <sup>8</sup>. Interestingly, the dorsal SVZ lacks high pathway activation but expresses the machinery needed for pathway activation <sup>8</sup>. To determine if hedgehog played a critical role in the production of dorsal-derived progeny, our lab removed the receptor Smoothed, a transducer of the pathway (unpublished). The removal of Smoothed compromised the production of both dorsal and ventral-derived progeny (unpublished). Previous studies in which SHH signaling was inactivated by the ablation of Smo or inhibition by pharmacological agents in the embryonic and post natal brain, has shown that there is a decrease in SVZ stem cell proliferation resulting in a decreased progenitor and neuroblast population<sup>2, 3, 16</sup>. A recent study was able to determine that the decrease in neurogenesis was due to the loss of Smo in the NSC population rather a direct effect on the microenvironment<sup>17</sup>. The authors attribute the loss of neurogenesis to the fact that there is decreased proliferation<sup>17</sup>. These results indicate that the hedgehog pathway is required for long-term maintenance of the NSC population. It also raises the question of why the dorsal-derived NSC require the hedgehog pathway but lack high pathway activation. Why does ectopic pathway activation respecify dorsal-derived neurons?

It has been previously suggested that in the dorsal region of the neural tube, Gli3 repressor regulates the fate specification of dorsal-derived progeny <sup>22</sup>. It has also been shown that there is more Gli3 mRNA in the dorsal region of the SVZ when compared to the remaining SVZ <sup>17</sup>. Our results indicate that not only is there an enrichment in Gli3, but there is also an increase in the repressor form of Gli3 in the dorsal region of the SVZ.

Next, we wondered if the increase had a functional role in the fate specification of the dorsal-derived progeny. To determine the role Gli3 played in dorsal identity, we removed the Gli3 gene from the dorsal NSC. We found that the progeny from these NSC no longer integrate into their normal superficial region but rather into the deep region of the granular layer. Dorsal NSC are not only responsible for the production of interneurons in the granular layer but are also the production of interneurons in the periglomerular layer. Our results indicate that the Gli3 repressor is required for the proper fate specification of dorsal-derived progeny.

Progeny of mutant Gli3 NSC, are similar to those of ventral NSC progeny. The shifts to a ventral-like phenotype suggest that in the absence of Gli3, Gli2 drives the activation of the hedgehog pathway in dorsal NSC. To determine whether Gli2 drives the fate of dorsal-derived progeny in the absence of Gli3, we ablated Gli2 and Gli3 in our double mutant animals. We found that in the absence of both Gli2 and Gli3 that dorsal-derived progeny no longer integrate into the deep region of the OB. Interestingly we found that the progeny now are integrated back into the superficial region of the OB, similar to that of wildtype littermates. However, the ablation of Gli2 and Gli3 was unable to restore the dorsal-derived PGC population. These results suggest that dorsal NSC

may require a distinct level of pathway activation for the production of PGCs versus granular layer interneurons. Alternatively, it maybe that the change observed in the granular layer and lack of TH expression in the PGC is due to a stunt in maturation.

The SVZ is a mosaic niche of NSC, which produces distinct immature neurons depending on the location of origin<sup>8, 10</sup>. The immature neurons that arise from the ventral, medial, and dorsal region integrate into different regions of the OB and are functionally different. It is now known that a molecular mechanism responsible for the diversity of immature neurons that arise from the SVZ is the hedgehog pathway<sup>8, 17</sup>. A previous paper from our lab has shown that the ventral region relies on high pathway activation for the production of progeny. However, It has also been shown that the dorsal region lacks high pathway activation, but requires the proper machinery<sup>8, 17</sup>. We show that removal of proteins that inhibit the hedgehog pathway allows stem cells to be redirected to a different fate.

## CHAPTER 2

### DEVELOPMENT AND VALIDATION OF A HIGH THROUGHPUT NEURAL STEM CELL CULTURE SYSTEM

#### Introduction

Neurogenesis has been a subject of intense research during the last decades. A central question is the elucidation of signaling events and transcriptional events that control the decision between proliferation, differentiation, and fate determination of neuronal stem cells and their subsequent progeny. Much research in the field of neurogenesis has focused on characterizing the progeny generated by the neural stem cells isolated in culture<sup>10, 18</sup>. However, which signaling pathways are important for the maintenance and promotion of fate determination has not yet been investigated in culture.

As a model system to investigate the pathways required for the regulation of neuronal progenitor cells proliferation and differentiation, we used NSC that were micro-dissected from the dorsal and ventral region of the SVZ. The culture system was a 96 well based approach that recapitulated the model used by<sup>10</sup>. In the future, we plan to use this approach to introduce perturbations to various developmental pathways and/or key proteins. We used this system to specifically analyze the changes that occur in the NSC and their subsequent progeny.

## Experimental Procedures

### Animals

The following mice lines were used and genotyped as previously described: CD1 WT, Gli3lox (Huang et al Dev Dyn, 2008.) and Ai14lox (Jackson Labs). Mice of either sex were used for the analysis. Experiments were performed in accordance to protocols approved by Institutional Animal Care and Use Committee at Vanderbilt University.

### Cell Culture

SVZ cells from postnatal day 1 (P1) (Timed pregnant CD1 wild-type females, The Harlan Laboratory or Gli3 conditional mice, in house) were placed in DMEM/F-12 (DF) containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml Gentamicin. Extracted tissues were triturated in 0.25% trypsin (15 min, 37°C, pH 7.3) and placed overnight in 24 well plastic tissue-culture dishes in N5 medium [DF containing N2 supplements, 35 µg/ml bovine pituitary extract, 5% FBS (Sigma), and 20 ng/ml EGF and basic FGF]. Unattached cells were collected, gently triturated, and replated onto uncoated plastic dishes, and grown to confluency in N5 media was added every other day. To induce differentiation, the cells were plated on glass 8 well chamber slides coated with 0.1 mg/ml poly-D-Lysine (Sigma) and 1 µg/ml laminin at densities of  $\approx 5 \times 10^4$  cells per cm<sup>2</sup>. The cells were proliferated to 90–100% confluency and were induced to differentiate by removing growth factors from the culture media. Neurons were allowed to differentiate in N6 media [DF containing N2 supplements, 35 µg/ml bovine

pituitary extract, and 2.5% FBS].The media were changed every other day. For immunostaining, cultures were fixed with 4% paraformaldehyde.

### Immunostaining

Immunostainings were carried out on paraformaldehyde-fixed cells according to standard procedures. Cells were blocked with 1% goat serum plus 0.10% Triton X-100(Sigma) in PBS for 30 min at 25 C, before primary antibody incubation at 4 C overnight. Primary antibodies used were mouse anti-GFAP (1:1000, Chemicon), chicken anti-GFAP (1:1000, Abcam), rabbit anti-doublecortin (1:1000, Cell Signaling), Guinea pig anti-doublecortin (1:1000, Cell Signaling), rabbit anti-tyrosine hydroxylase (1:500, Pel-Freez Biologicals), monoclonal anti-calbindin D28k(1:500, Sigma), rabbit anti-calretinin(1:500, Swant), rat anti-Ki67(1:1000, Novocastra), mouse anti- $\beta$ III tubulin(1:1000,Millipore), and rabbit anti-calbindin D28k (1:1000, Swant). The secondary antibodies used were conjugated to AlexaFluor dyes (1:1000, Invitrogen/Molecular Probes) and nuclei were counterstained with DAPI (Sigma).

### Microscopic Analysis and Quantification

Fluorescent staining in the eight well chamber slide (Millipore) was visualized using a LSM 710 Meta Inverted confocal microscope. Fluorescent staining in the BD 96 well imaging plate was visualized using the CellAvisa (Dynamic Devices) and analyzed the data using a MATLAB script developed by Stephen Hummel (Quaranta lab, MS in prep.). Data were quantified and analyzed using GraphPad Prism 5.

## Proliferation of Neural Stem Cells Decrease with Differentiation

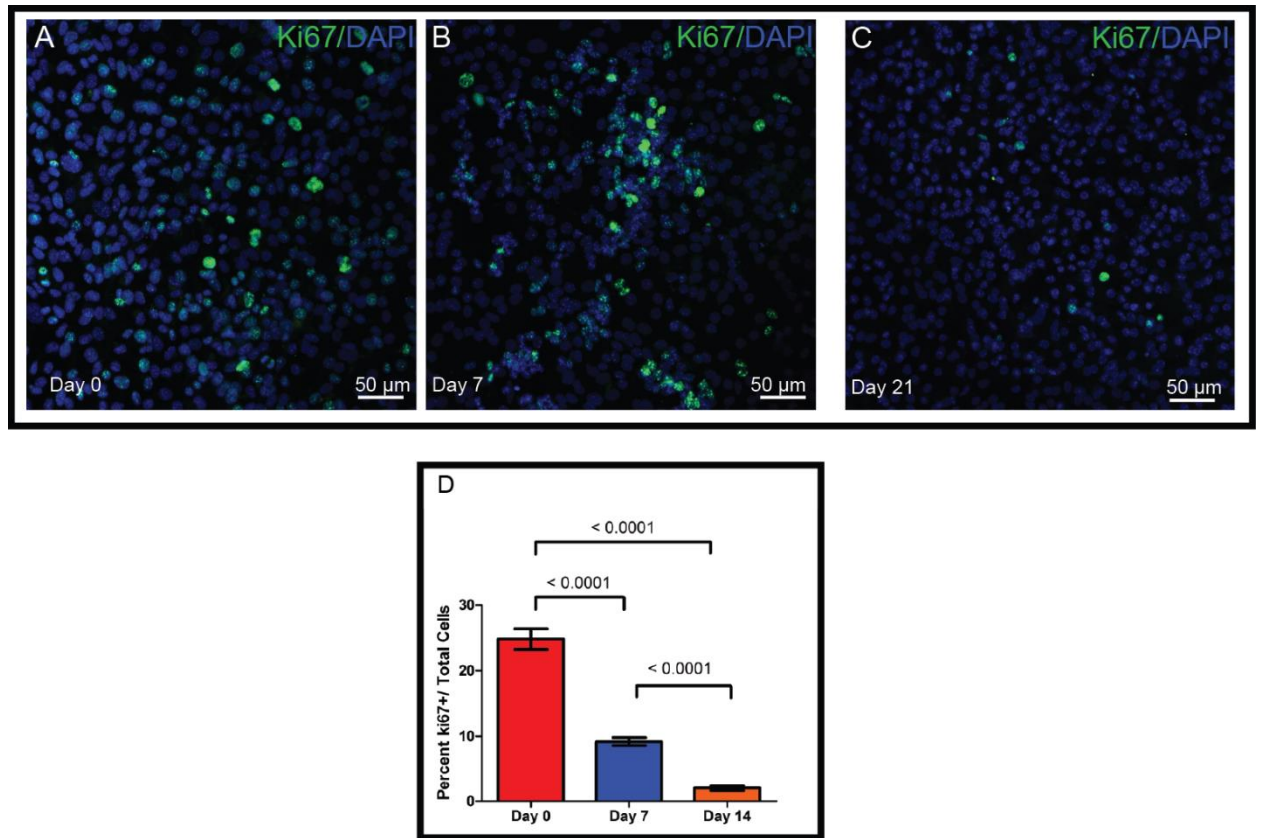


Figure 5: Proliferation of Neural Stem Cells Decreases with Differentiation. During initial plating of dissociated SVZ derived tissue, a mitotically active, multipotent cell emerges in vitro. Immunofluorescence analysis of Ki67 expression of NSC maintained in growth media (5A). Removal of mitogenic stimuli (FGF and EGF) from the growth media results in the differentiation of NSC, and thereby decreases the ability of the NSC to divide Figure (5B-C). (5D). Quantification of the percent of Ki67 positive cells after mitogenic stimuli removal. When maintained in growth media, over 25% of the cells are proliferating. Upon the removal of mitogenic stimuli, the number of proliferating decreases from 10% at day 7 to 1% at day 21. Scale bars, 10 µm.

## Neural Stem Cells Remain Multipotent in Culture

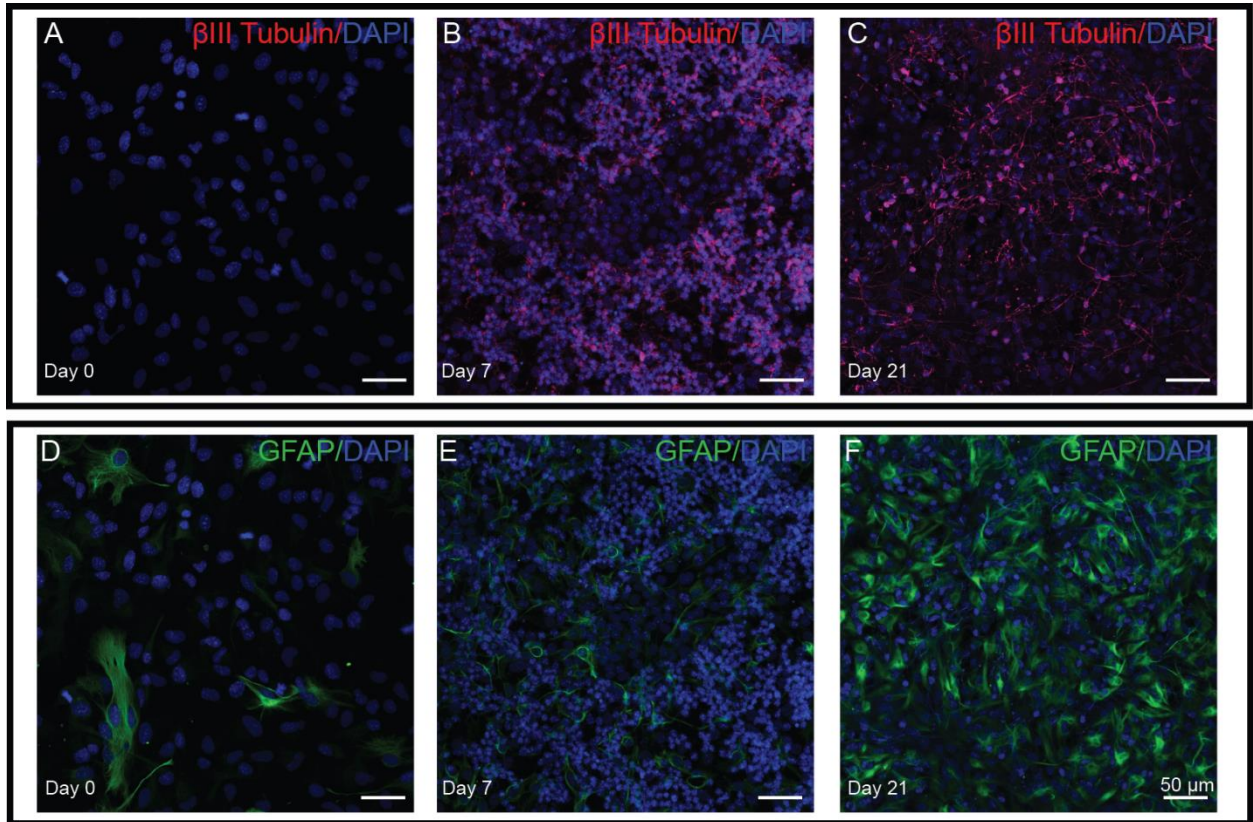


Figure 6: Neural Stem Cells Remain Multipotent in Culture. Consistent with prior publications, monolayers of SVZ-derived NSC can be induced to differentiate into neuroblasts and astrocytes when maintained *in vitro*. During initial removal of mitogenic stimuli the cultures are negative for an interneuron marker (6A), but have a population of GFAP + cells (6D). At day 7, abundant numbers of interneuron colonies begins to express  $\beta 3$ -tubulin, which is a marker of maturing neurons figure (6B). Expression of the mature neuronal marker  $\beta 3$ -tubulin persists until day 21 figure (6C). During day 7, there is a drastic increase in the GFAP + cell population, which continues until day 21 figure (6E-F). The number of days indicates time after the induction of differentiation. Scale bars, 50  $\mu$ m.



## Maturation of NSC to Region Specific Progeny

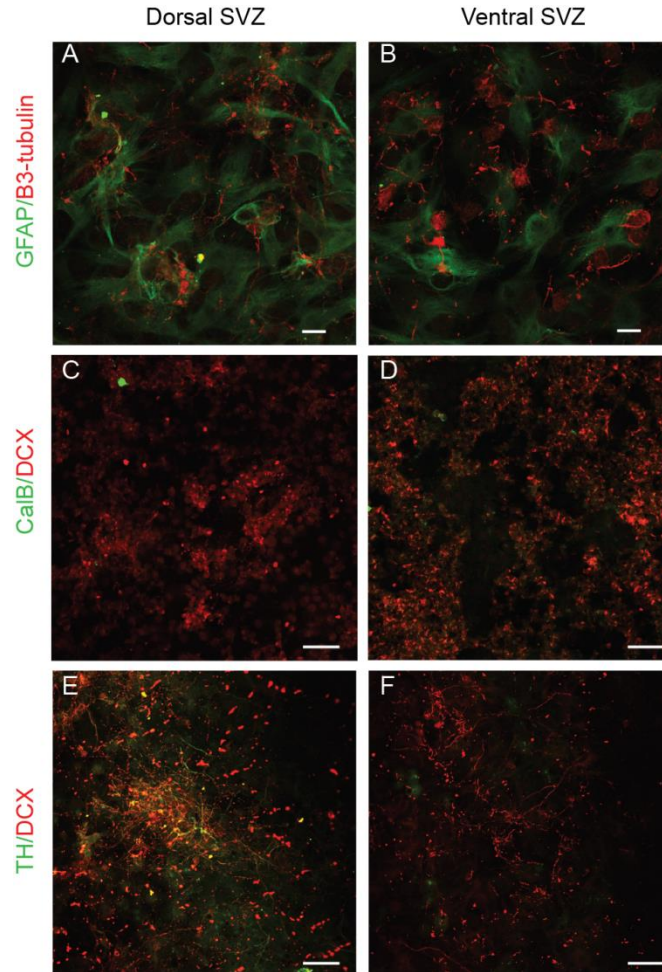


Figure 7: SVZ-derived NSC maintain their region-specific potential in culture. Confocal microscopy analysis of differentiated NSC cultures immunostained to identify neurons (Dcx+), and astrocytes (GFAP+) after 21 days of differentiation. Cultures derived from dorsal SVZ, and ventral SVZ generate these two cell types figure (7A-B). When stained for neuron type specific markers Calbindin (7C-D), and Tyrosine hydroxylase(7E-F) cultures exhibited region-specific neuron production, with most of the TH+ cells being produced by Dorsal SVZ , and most of the CalB+ cells produced in the Ventral SVZ (7I). Scale bars, 10  $\mu$ m in Figure (7A-B) and 50  $\mu$ m in Figure (7C-F) .

## High throughput Imaging of NSC Maturation to Progeny

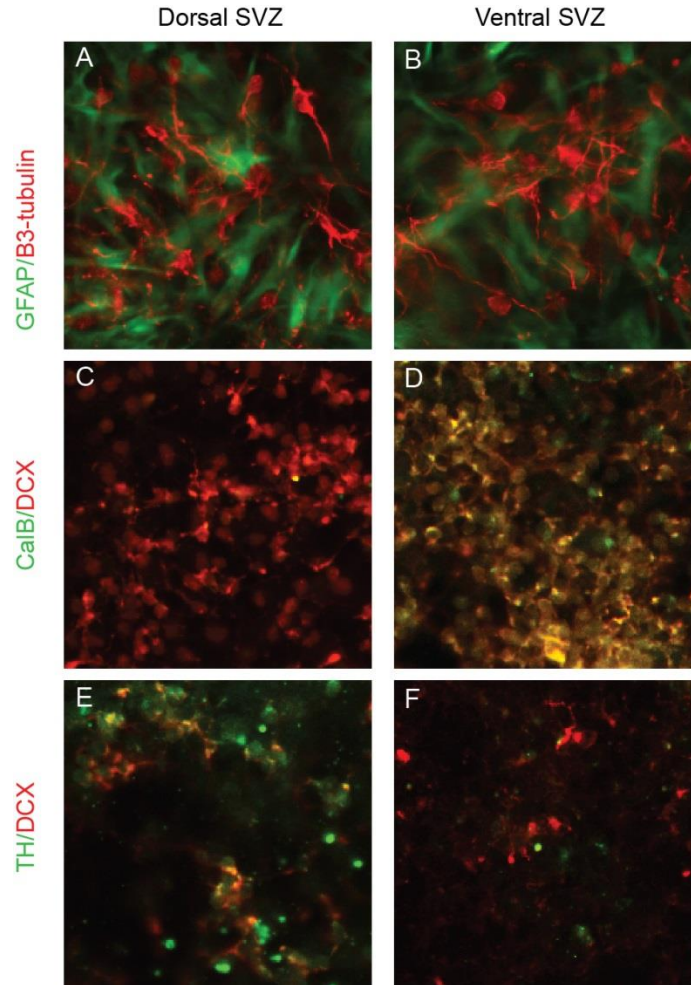


Figure 8: High throughput imaging of NSC maturation to prospective progeny. SVZ-derived NSC maintain their region-specific potential in culture. High throughput imaging analysis of differentiated NSC cultures immunostained to identify neurons (Dcx+), and astrocytes (GFAP+) after 21 days of differentiation. Cultures derived from regions Dorsal SVZ, and Ventral SVZ generate these two cell types figure (8A-B). When stained for neuron type specific markers Calbindin (8C-D), and Tyrosine hydroxylase(8E-F) cultures exhibited region-specific neuron production, with most of the TH+ cells being produced by Dorsal SVZ , and most of the CalB+ cells produced in the Ventral SVZ (8D). Imaged with the 40x objective on the Cellavista.

## Results

### Proliferation of Neural Stem Cells Decreases with the Induction of Differentiation

To expand the SVZ NSC population so that neurogenesis could be studied *in vitro*, single-cell suspensions of micro-dissected SVZ tissue were grown in a monolayer culture. These cells NSC remain proliferative when grown in the presence of N5 growth media (described above) (Figure 5A). To study neurogenesis we induced differentiation of the culture by removing the growth factors EGF, FGF, as well as decreasing the concentration of FBS<sup>18</sup>. As the culture undergoes differentiation the number of cells in the culture increases and the number of Ki67 positive cells decreases by day 7 (Figure 5B). We analyzed the cultures after 21 days, because this should allow for full maturation of NSC progeny, so we were not surprised to see that by day 21 proliferation was largely absent (Figure 5C).

### Neural Stem Cells Remain Multipotent in Culture

Neurogenesis in the mouse brain takes about 28 days, so we continued the culture for 3 weeks to allow for full maturation<sup>10, 18, 19</sup>. During day 0 of differentiation neuronal marker  $\beta$ III-tubulin was not detected (Figure 6A). Instead, we see that there is a population of GFAP-expressing NSC present (Figure 6D). The removal of growth factors and serum induced differentiation of the monolayer culture, leading to an increase in colonies of  $\beta$ III-tubulin positive neurons and mature GFAP-expressing astrocytes by day 7 (Figure 6B-E). Further examination of the culture at day 21 showed the increased expression of  $\beta$ III-tubulin in the immature neuron population and full development of the mature GFAP-expressing astrocytes (Figure 6C-F).

## Maturation of Neural Stem Cells to Region Specific Progeny

Our results indicate that SVZ-derived NSC remain neurogenic in culture, and upon induction of differentiation they produce astrocytes and neurons (Figure 7A-B). The neurons produced from the SVZ are specifically programmed from the region in which they are derived. Merkle et al has shown that cultured NSC retain their region specific identity<sup>10</sup>.

Next, we investigated whether our NSC and their subsequent progeny retain their regional specific identity in culture. Micro-dissected dorsal and ventral SVZ were expanded separately and differentiated into neurons for 21 days. These neurons were fixed and stained for markers that were specific for ventral, dorsal, and medial derived neurons. Dorsal and ventral derived neurons in the olfactory bulb express Tyrosine hydroxylase and Calbindin respectively as previously reported <sup>8, 10</sup>. We found that ventral derived NSC produce neurons that express calbindin similar to those reported by Merkle et al<sup>10</sup>. We also found that dorsal-derived NSC produce neurons that express Tyrosine hydroxylase similar to prior reports <sup>10</sup>.

## High-throughput Imaging of Neural Stem Cell Maturation to Prospective Progeny

Detection of neuronal cell differentiation is important for the studying of cell fate decisions under various stimuli and or environmental conditions. Due to the large colonies of immature neurons generated by the culture system, we employed a high throughput approach to analyze the maturation of the culture. The Cellavista (Dynamic Devices) was used to analyze cultures derived from the dorsal and ventral SVZ. These cultures were differentiated for 21 days and fixed for analysis.

First, we used the cellavista to determine if the NSC from the dorsal and ventral SVZ cultures remain multi-potent in culture. We found that the dorsal and ventral derived NSC are multi-potent by their expression of GFAP (mature astrocytes) and doublecortin (immature neurons) Figure (8A-B).

Next, we wanted to determine whether the NSC's subsequent progeny retain their regional specific identity in culture. Dorsal and ventral derived NSC were expanded separately and differentiated into neurons for 21 days. These neurons were fixed and stained for markers that were specific for ventral, dorsal, and medial derived neurons. We found that ventral derived NSC produce neurons that express calbindin Figure (8 C-D) and dorsal- derived NSC produce neurons that express Tyrosine hydroxylase Figure (8 E-F) similar to those reported by <sup>10</sup>.

## Discussion

The mammalian brain actively undergoes neurogenesis; progeny from the SVZ mature in the olfactory bulb<sup>10, 18</sup>. It is known that the immature neurons take 28 days to migrate to the olfactory bulb and integrate into the circuitry<sup>10, 18</sup>. We demonstrated feasibility of inducing and detecting neurogenesis over a time span of multiple weeks by confocal and high throughput imaging. Cultures were created from SVZ derived dorsal and ventral NSC and were seeded in either in eight well or 96 well plates<sup>10, 18</sup>. Differentiation of these cultures was induced following mitogen withdrawal. The cultures were analyzed for changes in proliferation as the culture underwent differentiation. We found that the rate of proliferation drastically decreased during maturation of the culture. These results are in line with the data from the original developers of the NSC culture system, in which progeny exit the cell cycle in order to differentiate <sup>18</sup>.

Next, we wanted to determine which types of neurons were generated during differentiation. Previous articles tracking NSC progeny *in vivo* and *in vitro*, have found that they differentiate into neurons, oligodendrocytes, and mature astrocytes<sup>10, 18, 19</sup>. We found that similar to the previous *in vivo* studies, the dorsal and ventral NSC that we micro-dissected from the SVZ, remain multi-potent *in vitro* and are able to generate neurons and mature astrocytes<sup>10</sup>.

Previous studies have found that neurons generated from the SVZ differentiate into distinct neurons depending on their site of origin<sup>10</sup>. It is known that neurons that are generated from dorsal NSC are known to express tyrosine hydroxylase, whereas ventral NSC derived neurons express calbindin<sup>10</sup>. We analyzed the dorsal and ventral derived neurons to determine if they retained their intrinsic identity. We found that the dorsal and ventral derived neurons express tyrosine hydroxylase and Calbindin respectively.

NSC and their subsequent progeny were first extensively identified and confirmed within the neurosphere *in vitro* culture system, however functional characterization of pathways required for fate specification have not been explored. It is therefore crucial that the *in vitro* methodologies designed to study NSC and subsequent progeny be able to maintain stemness and generate a large number of neurons. We have been able to show that the NSC isolated from the SVZ of mice, expanded in our system, were differentiated into the proper neurons. The culture system would provide a robust, retrospective read-out that would allow the investigation of function, regulation, and frequency of NSC and their subsequent progeny.

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